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An Optimized Microplate Assay System for Quantitative Evaluation of Plant Cell Wall-Degrading Enzyme Activity of Fungal Culture Extracts

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ABSTRACT: Developing enzyme cocktails for cellulosic biomass hydrolysis complementary to current cellulase systems is a critical step needed for economically viable biofuels production. Recent genomic analysis indicates that some plant pathogenic fungi are likely a largely untapped resource in which to prospect for novel hydrolytic enzymes for biomass conversion. In order to develop high throughput screening assays for enzyme bioprospecting, a standardized microplate assay was developed for rapid analysis of polysaccharide hydrolysis by fungal extracts, incorporating biomass substrates. Fungi were grown for 10 days on cellulose- or switchgrass-containing media to produce enzyme extracts for analysis. Reducing sugar released from filter paper, Avicel, corn stalk, switchgrass, carboxymethylcellulose, and arabinoxylan was quantified using a miniaturized colorimetric assay based on 3,5-dinitrosalicylic acid. Significant interactions were identified among fungal species, growth media composition, assay substrate, and temperature. Within a small sampling of plant pathogenic fungi, some extracts had crude activities comparable to or greater than *T. reesei*, particularly when assayed at lower temperatures and on biomass substrates. This microplate assay system should prove useful for high-throughput bioprospecting for new sources of novel enzymes for biofuel production.

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KEYWORDS: bioethanol; biomass conversion; cellulase; cell wall hydrolysis; high throughput screening; plant pathogens

Introduction

Hydrolysis of the plant cell wall polysaccharides cellulose and hemicellulose to fermentable sugar monomers is a critical step in the conversion of lignocellulosic material to ethanol (Jørgensen et al., 2007; Merino and Cherry, 2007). The most effective means of lignocellulosic hydrolysis uses a cocktail of different cellulolytic enzymes, but conversion is still not optimal (Berlin et al., 2007; Cherry and Fidantsef, 2003; Irwin et al., 1993; Jeoh et al., 2002; Kabel et al., 2006). There is a need to develop more effective cocktails with a range of properties complementary to current cellulase systems, at a significantly reduced cost. Multiple reports have underscored this necessity in order to reduce ethanol production costs from 10–25 cents to 3–5 cents per gallon (Gray et al., 2006; Jørgensen et al., 2007; Lin and Tanaka, 2006; USDOE, 2004, 2005, 2006; Zhang et al., 2006). New cocktails must be adaptable to a wide range of cellulosic feedstocks, from dedicated energy crops to agricultural and forestry residues, depending on regional feedstock availability. A diverse library of cellulolytic enzymes will allow development of cocktails specific to any available feedstock.

Trichoderma reesei (teleomorph = *Hypocrea jecorina*) is the major industrial source for cellulases and hemicellulases. However, genome sequencing and analysis have revealed

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that it contains the smallest number of cell wall degrading enzymes (CWDEs) when compared to those found in 13 fungal genomes (Martinez et al., 2008). For example, the phytopathogens *Magnaporthe grisea* and *Fusarium graminearum* (teleomorph = *Gibberella zeae*) contain many more cellulases, hemicellulases, pectinases, carbohydrate binding modules, carbohydrate esterases, and polysaccharide lyases in comparison to *T. reesei* (Martinez et al., 2008). Some phytopathogens, such as *G. zeae*, produce complete systems for biomass hydrolysis including endocellulases, exocellulases, β-glucosidases, hemicellulases, and ferulic acid esterases (Paper et al., 2007; Phalip et al., 2005). In addition to having a greater total number of CWDE predicted from genomic analysis, *G. zeae* also possesses CWDE from several families of glycosyl hydrolases (GH 51, 53, 93) that are not represented in the *T. reesei* genome (Martinez et al., 2008; Paper et al., 2007; Phalip et al., 2005). From the genome comparisons to date, virulent plant pathogenic fungi appear to present a largely untapped resource for novel hydrolytic enzymes. The plant cell wall is the major barrier that pathogens must surmount for successful invasion of plant tissue (Annis and Goodwin, 1997; Aro et al., 2005; Esquerre-Tugaye et al., 2000; Mendgen et al., 1996; Tonukari, 2003; Toth and Birch, 2005). Because the living plant can mount defenses, plant pathogens need to rapidly penetrate the plant cell wall in order to survive and reproduce (Esquerre-Tugaye et al., 2000). Thus, plant pathogens must employ a range of plant CWDEs to quickly infiltrate into the cell and overcome the plant's defenses (Annis and Goodwin, 1997; Tonukari, 2003; Walton, 1994).

Certain plant pathogens may possess unique enzymes that might complement commercial enzyme preparations, resulting in faster and more complete biomass hydrolysis with less enzyme loading. Conventional substrates used for cellulolytic activity studies, however, are insufficient to fully evaluate a pathogen's plant cell wall degrading abilities since microorganisms can produce numerous enzymes with different catalytic modes of attack and substrate specificity (Tomme et al., 1995; Warren, 1996). Cellulases represent several types of enzymes that hydrolyze cellulose. These include exocellulases (EC 3.2.1.91), which release cellobiose from the ends of cellulose chains; endocellulases (EC 3.2.1.4), which attack randomly along cellulose chains yielding a variety of sugars and oligosaccharides and creating additional sites for exocellulase activity; and processive endocellulases, which randomly attach to cellulose and release sugars as they move along the microfibril (Coughlan, 1985; Warren, 1996; Wilson and Irwin, 1999). Cellobiose is the major product of exocellulases, and is inhibitory to their activity (Duff and Murray, 1996; Wen et al., 2004). Many cellulase systems employ β-glucosidase (EC 3.2.1.21) to hydrolyze cellobiose and other oligosaccharides produced by cellulases to glucose, reducing end product inhibition and increasing total fermentable sugar yield (Wilson, 2008). The products of the various cellulases can be translated into activities measurements by using different substrates to elucidate modes of attack (Ghose, 1987). The standard filter paper activity assay is a good measure of total cellulase activity since filter paper is a nearly pure cellulose substrate and is readily available, with an established and accepted assay method (Coward-Kelly et al., 2003; Ghose, 1987; Xiao et al., 2004; Zhang et al., 2006). Microcrystalline cellulose, such as Avicel, can be used to measure exocellulase activity because it has a low degree of polymerization and relatively low accessibility (Wood and Bhat, 1988; Zhang et al., 2006). Soluble cellulose substrates such as carboxymethyl cellulose (CMC) can be used to measure endocellulase activity, because CMC has a high degree of polymerization and is readily accessible for endocellulolytic cleavage, but methylation may block processive enzyme action (Ghose, 1987; Irwin et al., 1998; Wood and Bhat, 1988; Xiao et al., 2005; Zhang et al., 2006).

In contrast to pure cellulose, however, the plant cell wall is a heterogeneous matrix of polysaccharides with diverse composition and linkages, as well as aromatic compounds found in lignin, such that hydrolysis of plant biomass is much more complex than for pure cellulose (Berlin et al., 2005, 2007; Rose, 2003; Schmer et al., 2008). Commercial enzyme preparations comprising various *T. reesei* enzymes such as endoglucanases, cellobiohydrolases and β-glucosidases are often limited in their activity on lignocellulosic substrates, and activities determined on filter paper, Avicel, and CMC do not necessarily correlate with activities on biomass (Berlin et al., 2005; Chundawat et al., 2008). Complete hydrolysis requires relatively large quantities of the enzyme product and often must incorporate additional enzymes with different catalytic behaviors in order to better degrade lignocellulosic biomass (Rosgaard et al., 2006). A comparison of enzyme activities of 14 commercial products on filter paper, cellobiose, AZCL-dyed xylan and grass and wheat bran fractions found large differences in performance dependent on the substrate (Kabel et al., 2006). Due to the heterogeneous composition of plant biomass, the enzymatic components needed for hydrolysis do not fall neatly into any catalytic activity category. Efficient degradation requires endocellulase and exocellulase activities as well as activity associated with β-glucosidase, xylanase (EC 3.2.1.8, 3.2.1.37), esterase (EC 3.1.1.72, 3.1.1.73), and additional CWDEs (Berlin et al., 2007; Selig et al., 2008). Measuring enzymatic activity on both simple substrates and complex biomass substrates such as those that would be fed into a biomass conversion plant may provide more information regarding the activity profiles of enzyme mixtures than only looking at one or a few substrates.

Much work has been done to develop high throughput technologies for characterizing enzymes and biomass for hydrolysis. The conventional International Union for Pure and Applied Chemistry (IUPAC) filter paper assay (FPA) method for measuring hydrolytic activity has been automated by Decker et al. (2003) to reduce operator error and adapted to use small reaction volumes by Xiao et al. (2004). Berlin et al. (2005) developed a high through-put screening assay utilizing lignocellulosic substrates. A high-throughput

methodology for measuring the digestibility of lignocellulosic biomass has been developed by Chundawat et al. (2008) using crystalline cellulose (Avicel) and ammonia fiber expansion (AFEX) pretreated-corn stover as substrates and a bio-enzymatic assay to determine fermentable sugars. In this study, the methods developed by Xiao et al. (2004, 2005) using 3,5-dinitrosalicylic acid (DNS) were adapted to use biomass substrates to measure hydrolytic abilities of fungal plant pathogens.

The overall objective of this research was to develop a standardized method for quantitative high throughput characterization of prospective microbial sources for enzymes capable of hydrolyzing plant cell wall polysaccharides, with the inclusion of typical biomass substrates. Specifically, the effects and interactions of fungal species, growth media, and assay temperature on hydrolysis rates of different polysaccharide substrates were examined in order to develop a standardized methodology for evaluating a large number of isolates. The DNS reducing sugar assay was modified for small hydrolysis reaction volumes in 96-well plates and non-standard enzyme substrates in order to more closely represent the types of lignocellulosic biomass being proposed for bioethanol production.

Materials and Methods

Fungal Cultures

Fusarium oxysporum f. sp. *loti* Fo060NY-93 (isolated from a stem of birdsfoot trefoil, *Lotus corniculatus* cv. ''Empire''), *G. zeae* Gz014NY-98 (isolated from a spike of wheat, *Triticum aestivum*, and *Sclerotinia sclerotiorum* Ss001NY-83 (isolated from a stem of soybean, *Glycine max*) were obtained from the New York Field Crops Pathogen Culture Collection of G. C. Bergstrom (Cornell University). *T. reesei* RUT-C30 was obtained from G. E. Harman (Cornell University). Fungal spores and/or mycelium were stored in 20% glycerol at −80°C. Isolates were revived by plating onto 50 mm Petri dishes containing ¼ strength potato dextrose agar (PDA: 6 g potato dextrose broth (BD 254920), 16 g agarose, 1 L H_2O) and incubating at 25°C until colonies had covered the surface. Mycelial plugs (6 mm) were transferred to Petri dishes containing agar modified from ATCC cellulose medium 907 (0.5 g $(NH_4)_2SO_4$, 0.5 g L-asparagine, 1 g KH_2PO_4, 0.5 g KCl, 0.41 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2$, 0.5 g yeast extract, 16 g agarose, 5 g cellulose, 1 L H_2O). Two variations of this medium with different carbon sources were used: either pure microcrystalline cellulose (Avicel, FMC type PH-101, 50 μm) or switchgrass (*Panicum virgatum* cv. ''Blackwell,'' 15 + 4 year stands, Pawling NY, Dutchess County, milled to pass through a 20 mesh screen). Fungi were grown on Avicel (Avi) and switchgrass (SG) agar for 10 days at 25°C. In preliminary experiments, 10-day-old cultures had the highest levels of reducing sugars produced when measured with the DNS assay when compared to 3- and 7-day-old cultures regardless of culture media used. Cultures at 14 days showed no additional increase in reducing sugar production.

Extract Preparation

Two replicated Petri dish cultures of each species per media were combined for each extraction. This was repeated for five independent biological replicates. The initial PDA plug inoculum was removed, and the remaining cultures and agar were chopped and extracted in 25 mL of 0.1 M sodium acetate buffer with 0.02 M sodium chloride and 0.02% sodium azide at pH 5.5, for 2.25 h at room temperature to obtain a crude mixture of extracellular proteins. Extract aliquots were centrifuged in 2 mL microfuge tubes at 13,000 rpm for 5 min, and the resultant supernatants then added to a deep 2 mL, 96-well master plate (Whatman, 7701-5200, Maidstone, England). Aliquots of each extract were added directly to the plate. A second aliquot of each extract was passed through a syringe filter (0.2 μm, 13 mm HT Tuffryn membrane, Gelman Sciences Acrodisc (4454), Pall Corporation, East Hills, NY) prior to addition to the plate.

Hydrolysis Reactions

Hydrolysis reactions were carried out in 96-well flat-bottom plates (Corning Life Sciences 3370, Corning, NY). Each well contained 90 μL of carbohydrate substrate in 0.1 M sodium acetate buffer pH 5.5. Ninety microliters of extract was added from the master plate to each well for a total reaction volume of 180 μL. Six assay substrates were evaluated: (1) filter paper (FP, 7 mm disk of Whatman #1 filter paper, 1001070, Maidstone, England) and suspensions (20 mg/mL) of (2) Avicel (Avi, FMC type PH-101 (50 μm)), (3) corn stalk (CS, Department of Plant Pathology, Cornell University) 4) switchgrass (SG), (5) carboxymethylcellulose (CMC, low-viscosity Sigma C5678, St. Louis, MO), and (6) arabinoxylan (AX, from oat spelt, Sigma X0627). Biomass was milled using a Wiley mill to a 80 mesh screen size. One gram of each biomass sample was washed with 50 mL of ddH_2O prior to hydrolysis to remove soluble lignin and sugars that could react with DNS and increase background. To accurately transfer insoluble substrates to the 96-well plates, suspensions were continuously agitated in preparation reservoirs with a magnetic stir bar. Negative controls of each extract (without substrate) and substrate in buffer (without extract) were included on each plate to identify background reducing activity. After preparing the hydrolysis reactions, plates were sealed with aluminum film (Axygen Scientific Inc. PCRAS200, Union City, CA) and incubated at 22, 37, and 50°C. The CMC and AX plates were incubated for 2 h; FP and Avi plates were incubated for 20 h; and CS and SG plates were incubated for 80 h. Time points were selected to allow more complete hydrolysis and allow appropriate production of reducing sugars within the dynamic range of the standard curve used for calibration of

the DNS assay. Reactions were stopped by freezing at −20°C.

Protein Quantification

Protein concentration was measured in 96-well microplates using a Sigma bicinchoninic acid (BCA) kit following manufacturer's instructions (Sigma). Extracts were diluted 1:20 to achieve protein concentrations consistent with the assay's dynamic range for quantification, up to 20 μg/mL protein. Bovine serum albumen standards at concentrations of 0, 0.5, 5, 10, and 20 μg/mL were prepared in 0.1 M sodium acetate buffer pH 5.5 and replicated 3 times per plate. Plates were incubated at 60°C for 1 h and absorbencies were measured at 562 nm on a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

Reducing Sugar Quantification

The DNS reducing sugar assay was previously adapted to utilize a miniaturized 96-well microplate system (Decker et al., 2003; Xiao et al., 2004, 2005). These methods were further modified to use crude extracts and finely ground biomass samples as substrates. DNS reagent was prepared as previously described (Ghose, 1987; Miller, 1959). The DNS reagent is nonspecific and reacts with both five and six carbon reducing sugars. Although this assay does not allow discrimination among specific carbohydrates, it can be used to quantify hydrolysis of a wide range of polysaccharide substrates. Glucose standards of 0, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/mL were prepared in 0.1 M sodium acetate buffer pH 5.5 and replicated three times per plate. Sixty microliters of standards or reaction hydrolysate was added to 120 μL DNS reagent in a 96-well PCR microplate (Axygen Scientific Inc. PCR-96) for a total reaction volume of 180 μL (Xiao et al., 2004, 2005). DNS reactions were carried out in PCR thermocyclers (Biorad iCycler, Hercules, CA and MJResearch Inc. PTC-100, Waltham, MA) by heating at 95°C for 5 min followed by cooling to 4°C for 1 min, and holding at 20°C. Thirty-six microliters of the completed DNS reaction was then added to 160 μL of ddH_2O in flat-bottom microplates (Corning Life Sciences 3370, Corning, NY) and absorbencies were measured at 540 nm.

Statistical Analysis

Data were analyzed using the statistical software package JMP (SAS Institute Inc., Cary, NC). To identify significant effects and interactions, data were fit to standard least squares models using the restricted maximum likelihood method. Experimental replicates and individual extracts were treated as random effects. For all responses analyzed, the effect of sample filtration and its interaction terms were not significant. Only unfiltered data was used for further analysis. The effects of fungal species (F), growth media (M), and their interaction were used to build a 4×2 factorial model for protein concentration (μg protein/mL extract). Log transformed reducing sugar concentrations (mM reducing sugar) had residuals with more normal distributions and equal variance than untransformed data. Insensitivity of the DNS assay and slight variation in the standard regression at very low reducing sugar concentrations resulted in estimation of a small number of activities less than or equal to zero. The minimum value was −0.14 mM. Log transformations are most effective when the smallest value is 1; therefore the range was shifted by adding 1.14 to all data. The effects of assay substrate (S), fungal species (F), assay temperature (T), growth media (M), and their interactions were used to build a $6 \times 4 \times 3 \times 2$ full factorial model for sugar concentration. All effects were treated as nominal. Non-significant interactions were removed to build the final model.

Results and Discussion

Standards for BCA and DNS Assays

All standard regressions for both protein and sugar measurements were linear. To account for plate to plate variation and allow more accurate comparisons across plates, each plate contained internal standards. A representative linear regression of measured protein concentrations versus known concentrations of protein standards yielded a slope of 1.081 and an R^2 value of 0.996. A representative regression of measured glucose concentrations versus known concentrations of glucose standards yielded a slope of 1 and an R^2 value of 0.998.

Protein Content

Fungal species, growth media, and their interaction had a highly significant effect ($P < 0.0001$) on protein content (Table I), as one might predict. In general, however, extracts from fungi grown on switchgrass media contained more protein than extracts grown on Avicel. There was greater species-to-species variation for Avicel-grown cultures than switchgrass-grown cultures (Fig. 1). *T. reesei* extracts had slightly more protein than the other species. The effect of growth media was not significant for *S. sclerotiorum* or *T. reesei*, while *F. oxysporum* and *G. zeae* produced more

Table I. Least squares model for the effects of fungal species (F) and growth media (M) on protein concentration of extracts (μg protein/mL extract).

Source	N_{parm}	DF	DF_{Den}	*F* ratio	*P*-value
F	3	3	28	32.48	<0.0001
M	1	1	28	28.27	<0.0001
F × M	3	3	28	12.54	<0.0001

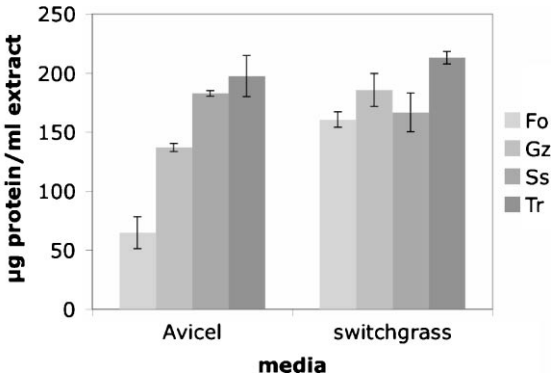

Figure 1. Protein content of fungal extracts when grown on Avicel and switchgrass media. Fo = *F. oxysporum*, Gz = *G. zeae*, Ss = *S. sclerotiorum*, Tr = *T. reesei*.

protein when grown on switchgrass. Limited conclusions can be drawn from these differences in protein content, however; in addition to the hydrolytic enzymes of interest, the total protein content of the extracts also includes protein from the growth media, non-cellulolytic extracellular proteins, and some intracellular proteins released by cell lysis during extraction. Because certain carbohydrate binding modules bind tightly to their insoluble substrates, the extraction methodology may not capture the entirety of a given culture's hydrolytic system. Weakly bound enzymes could be over-represented in the extracts, while tightly bound enzymes may remain attached to insoluble particles in the media. Use of detergents or high temperatures to enhance extraction of these tightly bound enzymes could significantly affect their activity however, so the extraction used in this study may be a limitation of capturing the full hydrolytic potential. Furthermore, it is difficult to determine the specific composition of the mixture of hydrolytic enzymes, especially when testing a large number of fungal species in a high-throughput system. Crude protein measurements are not useful in this context to calculate specific enzyme activities and kinetic data, because such calculations are most meaningful when combined with an estimation of the amount of specific enzymes in the mixture assayed (Zhang et al., 2006). In this study, activity is estimated only based on reducing ends produced over time from a single time point after the reaction had occurred for a suitable period for reducing sugar production. Extracts that demonstrate superior reducing end production will be further characterized to identify individual enzyme components in future studies.

Hydrolytic Activity

No reducing activity was detected in any of the negative controls. Quantification of reducing sugars using the DNS colorimetric reagent was highly reproducible both within a given extract (assay variation) and among replicated culture extractions (biological variation) (Table II). Most coefficients of variation (CV = σ/μ) were low. A few outliers had relatively high values, however these samples also had the smallest means, leading to inflation of calculated CVs. Most of the variation observed in this study was due to differences in fungal growth among cultures and extraction methodology, not from reducing sugar quantification. Excluding *F. oxysporum* tested on Avicel at 22°C, biological replicates had CVs less than 0.42 and experimental replicates had CVs less than 0.29. Variation among extract replicates was higher than among experimental replicates using a single extract, but results for both were highly reproducible.

Table II. Comparison of variation within (assay) and among (biological) extracts for five replicated measurements.

	Within extract						Among extracts					
	Fo A			Tr A			Fo A			Tr A		
	μ	σ	CV	μ	σ	CV	μ	σ	CV	μ	σ	CV
CMC												
22	0.36	0.01	0.03	0.81	0.04	0.05	0.40	0.16	0.41	0.94	0.30	0.32
37	0.41	0.01	0.03	0.96	0.03	0.03	0.39	0.06	0.15	1.03	0.08	0.07
50	0.52	0.03	0.06	1.09	0.03	0.03	0.56	0.07	0.13	1.20	0.12	0.10
Avi												
22	0.03	0.02	0.68	0.16	0.01	0.08	0.02	0.02	1.05	0.25	0.05	0.19
37	0.08	0.01	0.12	0.43	0.02	0.05	0.06	0.03	0.40	0.53	0.13	0.24
50	0.23	0.06	0.28	1.30	0.10	0.08	0.16	0.07	0.41	1.43	0.19	0.13
SG												
22	0.17	0.01	0.04	0.34	0.02	0.05	0.12	0.03	0.27	0.37	0.05	0.13
37	0.26	0.01	0.05	0.48	0.05	0.10	0.19	0.05	0.26	0.47	0.03	0.07
50	0.26	0.02	0.08	0.69	0.02	0.03	0.21	0.06	0.28	0.60	0.08	0.13

Mean µg reducing sugars/mL hydrolysate (μ), standard deviations (σ), and coefficients of variation (σ/μ) are presented for extracts of two fungi grown on Avicel medium, *F. oxysporum* (Fo A) and *T. reesei* (Tr A). Extracts were assayed on three substrates, carboxymethylcellulose (CMC), Avicel (Avi), and switchgrass (SG) at three temperatures, 22, 37, and 50°C.

The DNS assay has been criticized for its use in characterizing complex mixtures resulting from hydrolysis of lignocellulose. Although results from DNS correlate well with results from HPLC for analysis of simple sugars, this is not necessarily the case for lignocellulosic hydrolysates (Rivers et al., 1984). DNS is influenced by the degree of polymerization of reducing oligosaccharides and interference with compounds such as tannins found in the hydrolysate and/or growth media (Breuil and Saddler, 1985; Rivers et al., 1984; Schwald et al., 1988). However, HPLC analysis can be time consuming and impractical for processing large numbers of samples in a high-throughput fashion (Chundawat et al., 2008). Despite its limitations, the DNS method is still used for rapid measurement of total reducing ends generated from a range of substrates, making it suitable for comparing relative activities of many samples using high-throughput screening methodology (Chen et al., 2008; Hu et al., 2008; Roman et al., 2008). The method adapted from Xiao et al. (2004, 2005) is quick, reproducible, and the materials required are relatively inexpensive and available in most laboratories. In this study, controls were

included to ensure that measured reducing sugars were a product of enzymatic polysaccharide hydrolysis, and not compromised by background interference from either the substrates or culture extracts. Upon identification of samples with promising CWDE systems, a more complete analysis of hydrolysis products using HPLC can be performed to determine specific polysaccharide production.

Individual activity measurements of polysaccharide hydrolysis under all conditions for five replicated culture extractions are given in Table III. Activity tended to increase with increasing temperature and more sugars were released from relatively simple substrates such as CMC and AX than from complex biomass substrates like SG and CS. *T. reesei* is generally a consistently superior producer of enzymes for hydrolysis, however, the difference in activity between *T. reesei* and the other fungi diminished when tested on more complex assay substrates and growth media. *T. reesei* RUT-C30, a hypersecretor of cellulases that is resistant to catabolite repression, does not appear to possess any new or altered enzymes than the parental strain QM6a (Ghosh et al., 1982; Sheir-Neiss and Montenecourt, 1984). Thus, although it produces large amounts of cellulase, total activity may be limited by its ability to degrade the complex and heterogeneous structure of plant cell walls. As previously described, activities of hydrolytic enzyme mixtures on relatively simple substrates such as filter paper, Avicel, and CMC does not necessarily correlate with activities on more complex substrates such as biomass (Berlin et al., 2005; Chundawat et al., 2008). This underscores the importance of identification and development of enzymes complementary to the *T. reesei* system, which is efficient for cellulose hydrolysis but less so for heterogonous polysaccharide substrates. Individual comparisons using unpaired *t* tests showed that when grown on switchgrass media and assayed at 37°C, *T. reesei* extracts had higher activity than *G. zeae* extracts on cellulosic substrates such as FP, Avi, and CMC ($P < 0.0001$) while *G. zeae* had higher activity than *T. reesei* on AX ($P = 0.0306$). There was no significant difference between the two species on CS ($P = 1.0000$) or SG ($P = 0.5047$). Switchgrass compared to Avicel growth media induced a significantly higher activity on AX at 37°C for *F. oxysporum* ($P = 0.0002$) and *G. zeae* ($P = 0.0508$), but not for *T. reesei* ($P = 0.4992$) or *S. sclerotiorum* ($P = 0.3799$). Switchgrass media also induced higher activities when tested on SG and CS for *F. oxysporum* ($P < 0.0001$ and $P = 0.0051$, respectively) and *G. zeae* ($P = 0.0018$ and 0.0632) but not for *T. reesei* ($P = 0.4520$ and 0.6685). Switchgrass cultures of *S. sclerotiorum* had lower activity on SG and CS than did Avicel cultures ($P = 0.0140$ and 0.0117). Activity on Avi was higher for *F. oxysporum* and *G. zeae* when fungi were grown on switchgrass ($P = 0.0075$ and 0.0431, respectively). However for *S. sclerotiorum* and *T. reesei* activity was higher when fungi were grown on Avicel ($P = 0.0004$ and 0.0052).

The least squares model identified the most significant effects and interactions. All four main effects (S, F, T, and M) were highly significant, as were several two and three factor

Table III. Mean rates of polysaccharide hydrolysis under all conditions for five replicated culture extractions.

			Activity on substrate μM reducing sugar released/minute					
Assay temperature	Growth media	Species	Avi	FP	CS	SG	CMC	AX
22	Avicel	*F. oxysporum*	0.04 (0.08)	−0.04 (0.04)	0.35 (0.15)	0.14 (0.04)	18.41 (7.60)	38.12 (23.51)
		G. zeae	0.09 (0.08)	0.01 (0.07)	0.49 (0.13)	0.28 (0.04)	20.56 (10.25)	60.43 (28.90)
		S. sclerotiorum	0.76 (0.22)	0.55 (0.15)	0.52 (0.09)	0.28 (0.00)	34.53 (10.93)	48.49 (21.42)
		T. reesei	0.99 (0.18)	0.78 (0.06)	0.69 (0.10)	0.42 (0.06)	43.51 (13.83)	67.38 (33.55)
	Switchgrass	*F. oxysporum*	0.10 (0.06)	−0.01 (0.07)	0.51 (0.07)	0.35 (0.06)	20.26 (7.41)	87.43 (42.90)
		G. zeae	0.15 (0.07)	0.06 (0.08)	0.60 (0.13)	0.45 (0.06)	22.52 (8.59)	97.48 (48.43)
		S. sclerotiorum	0.31 (0.12)	0.23 (0.08)	0.42 (0.09)	0.23 (0.05)	29.68 (11.8)	52.7 (23.68)
		T. reesei	0.72 (0.16)	0.49 (0.11)	0.67 (0.12)	0.43 (0.04)	41.65 (10.4)	70.85 (40.62)
37	Avicel	*F. oxysporum*	0.25 (0.11)	0.14 (0.08)	0.42 (0.13)	0.22 (0.06)	18.02 (2.73)	34.48 (6.15)
		G. zeae	0.38 (0.15)	0.31 (0.11)	0.57 (0.11)	0.39 (0.06)	19.30 (1.01)	56.02 (7.99)
		S. sclerotiorum	1.62 (0.07)	1.35 (0.10)	0.55 (0.08)	0.33 (0.03)	35.40 (4.27)	47.93 (7.42)
		T. reesei	2.28 (0.25)	1.78 (0.25)	0.76 (0.18)	0.55 (0.04)	47.62 (3.55)	61.62 (7.74)
	Switchgrass	*F. oxysporum*	0.46 (0.09)	0.29 (0.05)	0.70 (0.10)	0.48 (0.05)	20.62 (2.08)	89.91 (18.68)
		G. zeae	0.52 (0.13)	0.45 (0.07)	0.72 (0.11)	0.55 (0.05)	22.10 (2.98)	97.66 (21.59)
		S. sclerotiorum	0.81 (0.16)	0.91 (0.14)	0.42 (0.04)	0.26 (0.04)	29.08 (2.80)	51.48 (8.41)
		T. reesei	1.72 (0.18)	1.30 (0.13)	0.72 (0.09)	0.53 (0.04)	44.33 (5.26)	67.99 (13.23)
50	Avicel	*F. oxysporum*	0.67 (0.31)	0.61 (0.27)	0.40 (0.14)	0.24 (0.07)	25.92 (3.26)	48.23 (7.86)
		G. zeae	0.62 (0.27)	0.71 (0.18)	0.42 (0.15)	0.28 (0.05)	27.45 (3.65)	81.11 (13.49)
		S. sclerotiorum	1.97 (0.16)	2.56 (0.55)	0.24 (0.08)	0.15 (0.01)	39.71 (5.83)	60.18 (5.17)
		T. reesei	6.64 (0.89)	5.94 (0.91)	0.91 (0.23)	0.70 (0.09)	55.71 (5.56)	97.27 (21.97)
	Switchgrass	*F. oxysporum*	1.14 (0.12)	1.11 (0.10)	0.59 (0.17)	0.42 (0.07)	27.74 (3.33)	122.56 (14.84)
		G. zeae	0.85 (0.22)	0.79 (0.13)	0.54 (0.18)	0.41 (0.05)	29.28 (4.55)	135.85 (23.30)
		S. sclerotiorum	1.06 (0.20)	1.31 (0.26)	0.19 (0.06)	0.13 (0.01)	34.71 (5.17)	69.04 (12.50)
		T. reesei	5.42 (0.51)	4.70 (0.34)	0.76 (0.16)	0.67 (0.10)	52.08 (7.11)	107.08 (24.39)

Avicel (Avi) and filter paper (FP) were hydrolyzed for 20 h, corn stalk (CS) and switchgrass (SG) were hydrolyzed for 80 h, and carboxymethyl cellulose (CMC) and arabinoxylan (AX) were hydrolyzed for 2 h. Standard deviations are presented in parentheses.

Table IV. Least squares model for the effects of assay substrate (S), fungal species (F), assay temperature (T), growth media (M), and their significant interactions on log transformed data (mM reducing sugars + 1.14).

Source	N_{parm}	DF	DF_{Den}	*F* ratio	*P*-value
S	5	5	592	1861.77	<0.0001
F	3	3	28	263.44	<0.0001
T	1	1	592	354.40	<0.0001
M	1	1	28	20.74	<0.0001
S × F	15	15	592	60.06	<0.0001
S × T	5	5	592	74.73	<0.0001
S × M	5	5	592	37.47	<0.0001
F × T	3	3	592	40.42	<0.0001
F × M	3	3	28	56.06	<0.0001
S × F × T	15	15	592	5.67	<0.0001
S × F × M	15	15	592	3.07	<0.0001

interactions (Table IV). The T × M interaction was not significant, nor were higher order interactions containing this term. The interactions with fungal species, growth media, and assay temperatures group the six assay substrates into four major categories: (1) insoluble, primarily exo-glucanase hydrolyzed microcrystalline cellulose (Avi, FP), (2) insoluble, heterogeneous monocot biomass (CS, SG), (3) soluble, endo-glucanase hydrolyzed cellulose (CMC), and (4) miscible arabinoxylan (AX). Avicel-based growth media generally induced greater hydrolytic activity in *S. sclerotiorum* and *T. reesei* for all substrates except AX (Fig. 2A–E). Switchgrass-based media showed a slight induction of hydrolytic activity for these two species on AX (Fig. 2E). Switchgrass media induced higher activity compared to Avicel media in *F. oxysporum* and *G. zeae* on all substrates tested, particularly CS, SG, and AX (Fig. 2D–F).

Regulation of fungal CWDEs is controlled largely at the transcriptional level (Aro et al., 2005). Polysaccharides and smaller molecules derived from them can induce expression of genes coding for appropriate enzymes. Expression can also be repressed when those enzymes are not needed. Production of xylanases and other accessory CWDEs is controlled in part by the transcriptional activator Xyr1 in *T. reesei* and *G. zeae*, and the orthologous XlnR in *F. oxysporum* (Brunner et al., 2007; Calero-Nieto et al., 2007, 2008; Rauscher et al., 2006; Stricker et al., 2006). Growth media is known to influence microbial enzymatic diversity and activity (Duff et al., 1987). Recent proteomic studies of *G. zeae* found at least 25 potential CWDEs expressed in planta during fungal infection of wheat heads and over 49 potential CWDEs expressed when grown on hop cell wall (Paper et al., 2007; Phalip et al., 2005). By comparison, only nine potential CWDEs were expressed when *G. zeae* was grown on glucose (Phalip et al., 2005). These enzymes have important roles in plant-microbe interaction since at least 20 CWDEs have been identified as potential virulence factors in *G. zeae* (Cuomo et al., 2007). Similar results have been found in *S. sclerotiorum*; when grown on wheat bran, more xylanases were induced than when grown on filter paper or xylan (Olfa et al., 2007). For these reasons, when screening fungal cultures for novel enzymes it is important to consider the effect of growth media on protein induction.

The slight increase in activity observed in extracts of Avicel-grown *T. reesei* and *S. sclerotiorum* when assayed on the two biomass substrates tested is probably due to greater induction of cellulases, rather than induction of xylanases, which was not significantly different for the two media. In contrast, switchgrass-based media induced the species *F. oxysporum* and *G. zeae* to produce a suite of enzymes with higher activity on CS, SG, and AX (Fig. 2C, D, and F), but extracts from this media had little effect on hydrolysis of pure cellulosic substrates like Avi, FP, and CMC (Fig. 2A, B, and E). These data suggest that the increased sugar release by *F. oxysporum* and *G. zeae* extracts observed on biomass is due, at least in part, to xylanase activity. The hydrolysis products of hemicellulose react with DNS in the same manner as cellulose hydrolysis products, contributing directly to the total sugar content measured in the assay. Removal of hemicellulose could also improve the accessibility of cellulose microfibrils, resulting in higher cellulase activity. While hemicellulase activity clearly contributes to overall biomass hydrolysis, other mechanisms such as removal of lignin via peroxidase or esterase activity could also be induced by microbial growth on biomass. Although the plant pathogens *F. oxysporum* and *G. zeae* have lower cellulase activity compared to *T. reesei* (Fig. 2A, B, and E), when grown on switchgrass media, they have comparable activity to *T. reesei* on biomass and higher activity on arabinoxylan, potentially due to the expression of different classes or larger concentrations of enzymes. This demonstrates that non-cellulolytic enzymes are being produced that contribute to overall biomass hydrolysis. These enzymes could potentially be used to complement and improve industrial enzyme preparations produced by *T. reesei*.

Comparing the activities of these isolates grown on different growth media also illustrates the need to evaluate multiple growing conditions for enzyme activities. Growth and CWDE production may be reduced if the fungi cannot adequately degrade and utilize the complex polysaccharides. Furthermore, biomass may contain compounds that are inhibitory to fungal growth. By comparing activities produced on Avicel- and switchgrass-media with multiple substrates, we were able to note the differential activities that indicate presence of non-cellulolytic enzymes.

Extracts from all species showed increased hydrolysis rates with increasing temperature for the substrates Avi and FP (Fig. 3A and B). Activity of *F. oxysporum* and *G. zeae* was not detectable at 22°C. On biomass, activity of *T. reesei* increased with increasing temperature, while *F. oxysporum*, *G. zeae*, and *S. sclerotiorum* had highest activity at 37°C (Fig. 3C and D). *S. sclerotiorum* had similar activities at 22 and 37°C, and significantly reduced activity at 50°C. *F. oxysporum* and *G. zeae* had lower activities than *S. sclerotiorum* and *T. reesei* when tested on CMC (Fig. 3E), but higher activities than *S. sclerotiorum* and comparable activity to *T. reesei* on AX (Fig. 3F). On both of these substrates, there was no difference in activity at 22 and 37°C,

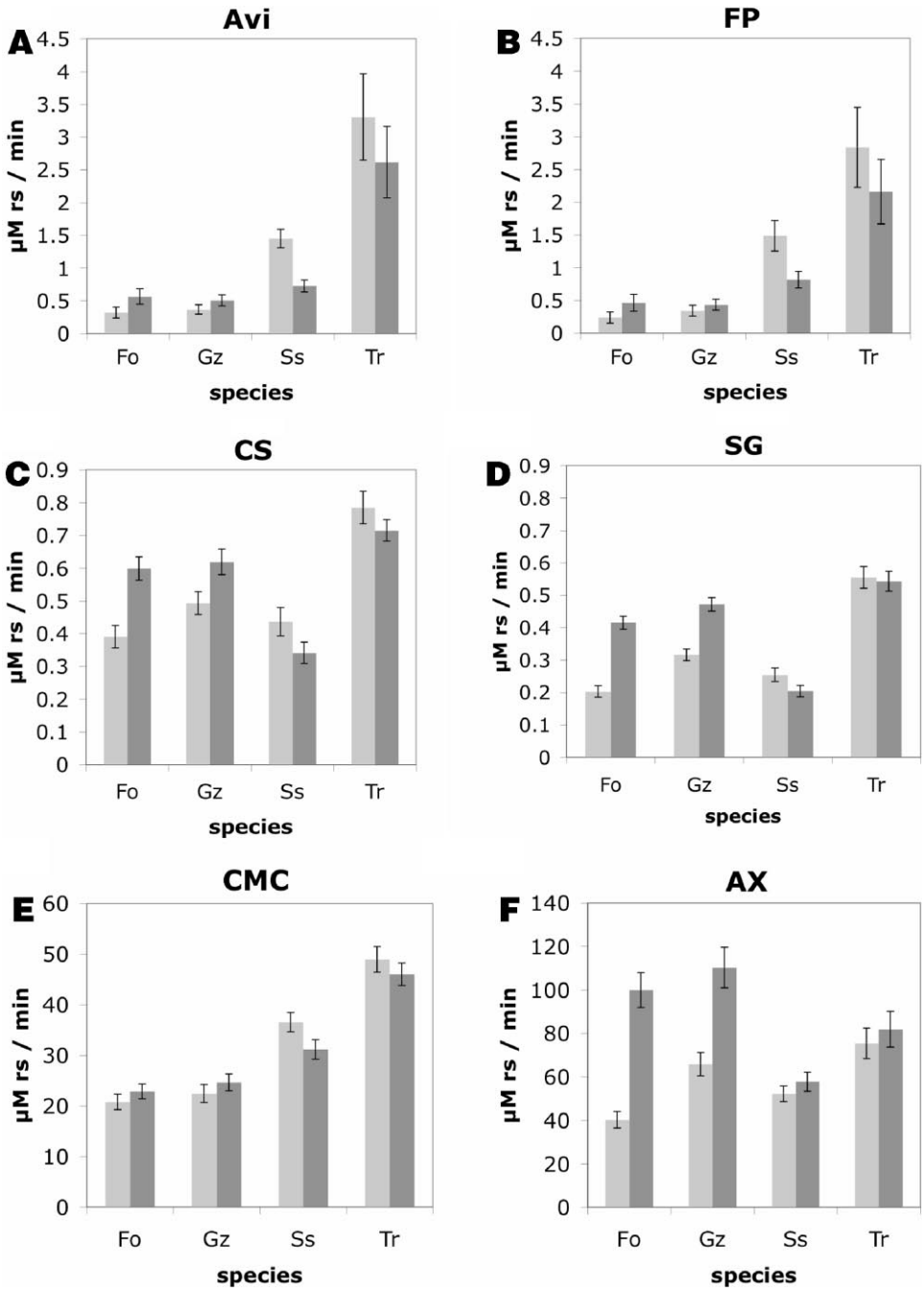

Figure 2. Effect of two growth media, Avicel (light gray) and switchgrass (dark gray), on polysaccharide hydrolysis rates by extracts of four fungi, *F. oxysporum* (Fo), *G. zeae* (Gz), *S. sclerotinia* (Ss), and *T. reesei* (Tr). Polysaccharide substrates include (**A**) Avicel (A), (**B**) filter paper (FP), (**C**) corn stalk (CS), (**D**) switchgrass (SG), (**E**) carboxymethylcellulose (CMC), and (**F**) arabinoxylan (AX). Avi and FP were hydrolyzed for 20 h, CS and SG were hydrolyzed for 80 h, and CMC and AX were hydrolyzed for 2 h. Error bars indicate mean standard error.

but increased activity at 50°C. Although arabinoxylanase activity is highest at 50°C, this assay was only conducted for 2 h, compared to 80 h for the biomass assay. Short-term improvements in activity by increasing temperature may be outweighed by the long-term consequence of thermal instability. The pathogens used in this study were isolated from New York State, and have the ability to thrive on plant biomass at moderate temperatures (Sutton, 1982). *G. zeae* overwinters on and within field residues of maize (*Zea mays*) and small grains, particularly in the recalcitrant nodal areas (Sutton, 1982). It grows as a saprophyte during the fall, winter, and spring, during which time CWDEs may be very important for growth and reproduction. There is evidence that temperatures between 22 and 24°C favor outbreaks of

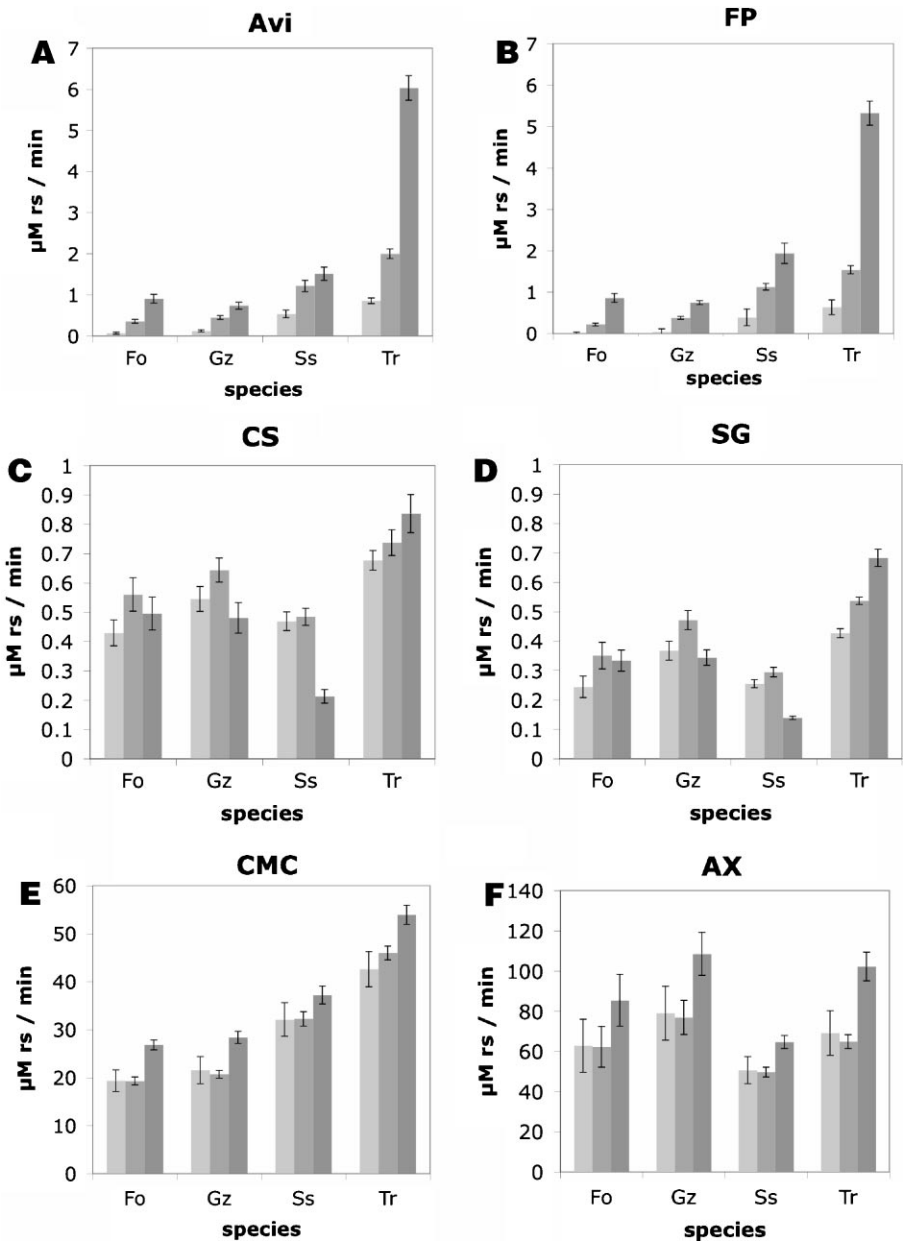

Figure 3. Effect of three temperatures, 22 (light gray), 37 (medium gray), and 50°C (dark gray), on polysaccharide hydrolysis rates by extracts of four fungi, *F. oxysporum* (Fo), *G. zeae* (Gz), *S. sclerotinia* (Ss), and *T. reesei* (Tr). Polysaccharide substrates include (**A**) Avicel (A), (**B**) filter paper (FP), (**C**) corn stalk (CS), (**D**) switchgrass (SG), (**E**) carboxymethylcellulose (CMC), and (**F**) arabinoxylan (AX). Avi and FP were hydrolyzed for 20 h, CS and SG were hydrolyzed for 80 h, and CMC and AX were hydrolyzed for 2 h. Error bars indicate mean standard error.

ear rot (Paul et al., 2007; Sutton, 1982). The fungus may benefit from copious production of CWDEs during this rapid necrotrophic growth phase. Similarly, although *S. sclerotiorum* is not a competitive saprophyte in the soil, it grows rapidly in culture and in planta and relies on CWDE to facilitate rapid colonization of plants (Hegedus and Rimmer, 2005; Morrall and Dueck, 1982).

Enzyme activity is expected to increase with increasing temperatures, in some cases doubling with every 10°C increase up until a point of thermal inactivation (Fullbrook, 1996; Rosgaard et al., 2006). Maximum activity for fungal cellulases has been observed between 45 and 55°C (Saddler and Gregg, 1998; Tengborg et al., 2001). Hydrolysis at higher temperatures with increased enzyme activity could reduce the costs associated with producing ethanol from biomass by reducing reaction time and required enzyme loading. Much work has been done to improve the thermostability of cellulases and other hydrolytic enzymes at temperatures above 50°C (Ai and Wilson, 2002; Gusakov et al., 2005; Voutilainen et al., 2007). In a conversion plant employing a

separate hydrolysis and fermentation scheme, each step can operate at its optimum temperature, making enzymes active at 50°C or higher important for efficient hydrolysis (Tengborg et al., 2001). On the other hand, plants operating with a simultaneous saccharification and fermentation schemes will require enzymes that are most active between 35 and 38°C for maximum hydrolysis (Szczodrak and Fiedurek, 1996; Tengborg et al., 2001). This is a compromise between the optimum temperature for hydrolysis and the optimum for fermentation, which is between 28 and 35°C (Szczodrak and Fiedurek, 1996). It is possible that plant pathogens that thrive in mesophilic environments may provide enzymes that could be optimized for either operational scheme, as is already the case with *T. reesei*.

Conclusions

This research presents a rapid, quantitative, reproducible, and high-throughput platform for analyzing CWDE activities of fungal extracts. By choosing appropriate cell wall components as substrates, it is possible to gain insight into the mechanism of hydrolysis for complex and heterogeneous biomass materials. Though this study used only two complex biomass substrates for analysis, more complete information on CWDEs substrate specificity and activity could be obtained by using additional biomass sources and pretreated biomass materials. Greater diversity of substrates will allow for better characterization and diversity of CWDEs for cellulolytic cocktails. Use of multiple fungal growth media and hydrolytic assay temperatures in this screening platform provides a more complete picture of substrate specificity and thermostability.

Microbes employ a variety of enzymes to deconstruct the plant cell wall and growth medium affects the activity observed in the final extracts. Complex polysaccharides such as those found in the cell wall can induce synthesis of enzymes preferentially hydrolyzing those sugars. In addition to substrate specificity, enzymes have temperature optima that may reflect the environment of the microbe that produces them. Enzymes of *T. reesei* are quite stable at higher temperatures, but improving thermal stability of novel accessory enzymes could be an important target for improving overall industrial biomass conversion. While the *T. reesei* enzyme system is quite efficient at degrading pure cellulose, there are opportunities to complement its enzyme systems with novel enzymes from other species. The fact that the plant pathogenic fungi used in this study compared favorably to *T. reesei* is supported by genomic and proteomic analyses demonstrating that plant pathogenic fungi are rich reservoirs of multiple classes of enzymes involved in cell wall breakdown (Martinez et al., 2008; Paper et al., 2007; Phalip et al., 2005; Yajima and Kav, 2006). Identifying superior species and isolates is an important first step to obtaining novel cellulolytic enzymes.

Future studies based on this initial work will characterize individual enzyme components produced by plant pathogens identified as having good crude activity to obtain information on mode of catalytic behavior and specific activity. With this information, more complete enzyme cocktails could be developed, incorporating additional cellulases and accessory enzymes complementary to current cellulolytic cocktails' activities. Plant pathogens present a unique opportunity for prospecting for novel cellulolytic and accessory enzymes for biomass hydrolysis.

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